Swami Ramanand Teerth Marathwada University
Nanded, Maharashtra, India

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Isolation and identification of carotenoid producing halophilic bacteria and archaea from solar salterns of Mulund (M.S.)

Research Proposal submitted in partial fulfillment of registration to Ph.D. Programme

In the subject Biotechnology faculty of Life science

Submitted by

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Thesis Title - Isolation and identification of carotenoid producing halophilic bacteria and archaea from solar salterns of Mulund (M.S.)

Introduction:-

From so many years microorganisms are isolated from the various sources like soil, water especially from the ocean. The increasing interest, in recent years, in microorganisms from hypersaline environments has resulted in the discovery of several new species and genera belonging to the Bacteria and Archaea domains. Halobacteria are a group of microorganisms forming a part of the domain Archaea that require high salt concentration for growth. Members of the Halobacteriaceae are dominant microorganisms in hypersaline environments worldwide including salt lakes, crystallizer ponds of solar slatterns, salt mines, as well as hypersaline soda lakes, haloarchaea are well adapted to hypersaline environments and require at least 1.5 M NaCl for growth.

Halophilic microorganisms have capacity to tolerate and live under high salt concentrations by generating a balance inside and outside of the cell to avoid osmotic stress. Further, accumulation of compatible solutes in cytoplasm such as glycine-betaine, sugar derivate like sucrose or trehalose and/ or inorganic ions such as sodium, potassium and chloride act as stabilizers of biologic structures. These traits of halophilic microorganism generate a great interest to the scientific community due to their potential applications in the biotechnological industries. Enzymes of halophilic microorganisms have advantage over their nonhalophilic counterparts due to their ability to maintain their stability and catalytic properties at high salt concentrations. Additionally, the halobacteria are reported to produce bacteriorhodopsin, bioplastics, and various enzymes their potential applications in different areas. However, extracellular enzymes production by halophilic microorganisms has not yet been investigated in detail. It is generating lot of interest in recent years on extracellular enzymes like proteases, amylases, DNases, pullulanases and lipases produced by halophilic microorganisms.

Explorations of coastal regions, solar salter by various researchers have yielded number of compounds that are useful in various sectors and products. In current project marine solar slatterns of Mulund (E) ($19^0$10’12’’N, $72^0$7’18’’E) Mumbai area has been proposed. The halophilic archaea are also capable of producing isoprenoid compounds such as carotenoid pigments.

Carotenoids Produced by Halophilic Archaea Carotenoids are liposoluble pigments responsible for the yellow-orange or orange-red colors of plants, algae, microorganisms, and animals. Animals cannot produce carotenoids but they obtain them from the diet [3]. The carotenoids bacterioruberin, carotene, lycopene, canthaxanthin, 3-hydroxy-echinenone, lycopersene, phytoene, phytofluene, and 2-isopentenyl-3,4-dehydrorhodopin have already been identified in halophilic archaea [1, 2]. Some of these carotenoids may be found at low concentrations, suggesting they may be precursors from other carotenoids. Usually, bacterioruberin is the main carotenoid from Halophilic archaea [1]. Halophilic archaea probably synthesize carotenoids through the mevalonate pathway. Bacterioruberin presents important biological functions in halophilic archaea. It acts as cellular membrane reinforcement since

it increases membrane rigidity and decreases water permeability. It also protects the microorganism from DNA damaging agents such as ionizing radiation, ultraviolet radiation, and hydrogen peroxide, probably due to its antioxidant capacity. Studies on the application of carotenoids produced by halophilic archaea are scarce. It is highly relevant to study halophilic archaea since these microorganisms have shown a high biotechnological potential and their carotenoids may be an alternative source of pigments.

Importance and use of carotenoids in food products :-

Commercial food products using carotenoids are expanding, and the greatest demand is in the Asian continent. The French cheese named vieux-pan contains the carotenoid produced by *Brevibacterium linens* due to its orange-red-brown color that improves the sensory quality of the product. In Russia, infant formulas are enriched with natural pigments such as lutein, which is present in breast milk, in order to improve children's health .Nutraceutical food products have also been applied in bakery products and pasta. In Japan, *Undaria pinnatifida* (wakame), edible seaweed rich in fucoxanthin, is commercialized as an ingredient for pasta. In India, a pasta containing fucoxanthin as an ingredient to improve its bio functional and nutritional qualities was developed. Besides the use of nutraceutical foods as a form of prevention and treatment of diseases, the administration of the bioactive compounds in their concentrated form is also a possibility for promoting health. The transport of carotenoids occurs from the intestinal mucosa to the blood vessels carried by lipoproteins. Carotenoids functional properties are related to reactions such as oxidation, reduction, hydrogen abstraction, and addition in biological membranes and their antioxidant power is fundamental for cell protection against free radicals and singlet oxygen formed in tissues.

Carotenoid Color Application : Activities References Lutein Yellow Poultry feed; functional nutrient Antioxidant Canthaxanthin Orange Poultry feed; fish feed; cosmetic Antioxidant, anticancer Lycopene Red Supplement in functional foods; additive in cosmetics Antioxidant, anticancer β-carotene Orange-red Nutraceutical; cosmetic; animal feed industries Antioxidant, anticancer, precursor of vitamin A Astaxanthin Pink-red Fish feed; cosmetic industry Antioxidant, photo protectant, anticancer, anti-inflammatory. Some carotenoids are precursors of vitamins, and they also present activities such as anti-inflammatory, antioxidant, immunomodulatory, anticancer, for cardiovascular therapy and neurodegenerative diseases, and anti-obesity. The carotenoids included as pro-vitamin A are β-carotene, α-carotene, and cryptoxanthin. Vitamin A is an essential nutrient for operation and maintenance of biological functions including vision, reproduction, and immunity. Beta-carotene is present in blood and tissues, which are associated with antioxidant activity and concomitantly with other carotenoids or antioxidants, can enhance their activity against free radicals. However, it can bring health risk at high doses. Carotenoids, acting as antioxidants eliminating free radicals, can modulate the risk of developing chronic diseases by inhibiting reactions mediated by reactive oxygen species (ROS). Reactive species are produced during cellular metabolism as a defense to infectious and chemical agents that may cause damage to DNA, proteins, and tissues,

contributing to the development of chronic diseases such as diabetes, Parkinson's, Alzheimer's, cardiovascular diseases, and cancer. In addition to the antioxidant properties, carotenoids exhibit anti-inflammatory activities owing to the protective effects of phytochemicals such as lutein and Astaxanthin. Astaxanthin has been shown to inhibit the production of pro-inflammatory mediators such as nitric oxide (NO) in macrophages, to increase the level of inflammatory cytokines, and to reduce oxidative stress. Neuroprotective effect, reduced neuroinflammation, improvement of insulin signals, and reduction of lipid levels were also verified. Inhibition of cell proliferation of colon cancer cells by the use of Neochloris oleoabundans carotenoids was observed, enabling its use as a functional food additive or nutraceutical with potential for the prevention of colon cancer. Beta-carotene, Astaxanthin, and capsanthin demonstrated ant proliferative effects on leukemic K562 cells. Studies indicated that the simultaneous use of different carotenoids was efficient against liver cancer. Patients were administered with β-cryptoxanthin-enriched mandarin orange juice and capsules of a carotenoids mixture-containing lutein, β-cryptoxanthin, lycopene, zeaxanthin, and fucoxanthin. Analyses of DNA array and protein-antibody array showed that the carotenoids interfered in the induction of genes.

Literature review:-

B.Ollivier *et al* 1994 isolated the novel thermophilic, halophilic, fermentative strictly anaerobic eubacteria from sediment of Tunisian Hypersaline Lake. According to them isolated species was phylogenetically similar with gram positive halophiles, gram negative thermophilic halophilic anaerobic bacteria and named that isolate as *Halothermothrix orenii*

Birgul Ozcan *et al* 2012 isolate halophiles from salt mine central Anatolia Turkey, isolated species belongs from four genera *Halobacterium, Halococcus, Haloarcula, Halorubrum* by using the 16s r-RNA analysis. Physicochemical analysis of water sample done with the help of Merc Spectroquant test kit and ICP-AES instrument.

A.Satbhai *et al* 2015 isolate 22 gram negative species of extremely halophilic microorganism and study the biotechnological applications like synthesis of proteases, PHA, Halocine and Bioemulsifire. The salt concentration of Dead sea shows 34.8% and absence of any microscopic living organism. Several Algal, Fungal, Archeal, Bacterial species isolated earlier from Dead Sea and *Dunaliella* species further studied commercially.

Sung-Taik Lee *et al* 2003 isolated denitrifying bacteria fom coastal sediment waste water contaminated with sea water in South Korea shows ability of degrading trimethylamine which is a common pollutant of sea water. Isolated two species kept for phylogenetic analysis of 16s-rDNA gives *α-Proteobacteria* and *Paracoccus species*. These shows strong affinity towards salinity and ability to produce enzyme hydrolases which degrades trimethylamine

Cumhur Cokmus *et al* 2007 isolated 33 halophilic archea from six hypersaline environment in Turkey. Isolated halophilic archea were gram negative and their 16s-rDNA analysis revealed that these isolates belonging from the *Halobacteriaceae* family and these are extremely halophilic archea.

Dr. F. Emerson Solomon and Dr. Kathir Viswalingam 2013 isolated two halotolerant bacteria from mangroves of Bhitarkanika coastal region of Orissa India. Which are gram positive rods and cocci, which may probably belongs to *Bacillus species* and *Micrococcus species* respectively. They found that the basis of halotolerence was changes the morphological behavior of isolates from rods to cocci.

Veena Uniyal *et al* 2014 isolate the novel halophilic strains of bacteria from Sahastradhara region Dehradun India. Collected sample kept for isolation on halophilic agar medium and halobacterium medium. Total eight strains of halophilic bacteria were isolated and studied for their application commercially.

Malini Basu *et al* 2016 isolated the halophilic bacteria from Sundarban mangrove soil. Isolated strains kept for salt tolerance which shows the moderate growth of isolated species at 20% Nacl concentration. Isolates were phylogenetically analysed by 16s r-RNA shows comparative sequence with *Halobacillus* species.

Archana Gaur *et al* 2015 isolated alkalophilic, moderately halophilic gram positive cocci from brine samples of Samber lake Rajasthan India; one of the hostile environment for the growth of organism due to high salinity and high pH 10. After morphological, biochemical and genetic analysis it was found that the isolated species may belong to *Saliniococcus roseus* strain NIOT bflm-S12 (99% 16s-rRNA homology). The isolate was able to tolerate Nacl level upto 20% with 12% Nacl level as optimum one. The pH range was found to be 7-11. It was producing hydrolyses like amylase, proteases, lipases and gelatinases which were active even at higher salt concentration.

Sumithra V *et al* 2015 isolated halophile from salt pan at Tuticorin district Tamilnadu using Sehgal and Gibbon agar medium, the isolate was capable of producing protease enzyme at high salt concentration 15%. The amount of protease estimated by Anson method was 0.51mg/ml and species activity about 1.12 u/mg.

Hiroshi Onishi and Koji Sonoda 1979 extracted, purified and analysed as extracellular α-amylase from *Micrococcus halobius* ATCC 21727. Isolated from unrefined solar salt. The enzyme was stable and active at higher temperature with optimum temperature 55^0c. But losses 20 hrs 40% its activity with increasing level of salts at same temperature.

After initial extraction of enzyme, the glycogen-amylase complex was formed and purified by DEAE-Cellulose column chromatography and Bio gel P-200 gel filters. Study on effect of pH and temperature on enzyme activity that enzyme has optimum pH 6.5 and optimum temperature 55^0c. On characterization of enzyme it was found that it has molecular weight 89000. The end product of enzyme catalyzed reaction on starch was maltose, maltotriose, maltotetrose and lesser amount of glucose.

E.H.Nabti *et al* 2013 isolated halotolerant *Bacillus licheniformis* and *Bacillus sp* from soil of Algeria. This shows antifungal activity against phytopathogens and shows PGPR, siderophore production, IAA production, some enzyme production. These are needed for the beter crop yield. Isolated species

detected phylogenetically by 16s-rDNA sequencing. Total nine phytopathogenic fungi used for detection of antifungal activity.

R.M. Gulve and A.M.Deshmukh 2012 isolated 107 marine *Actinomycetes* from near sea shore sediment and sea water from Konkan coast of Maharashtra India. Out of 107 strains only 8 strains shows antagonistic against *Bacillus subtilis, Staphylococcus aureus, Proteus vulgaris, Escherichia coli, Klebseilla pneumoniae, Pseudomonas aeruginosa, Candida albicans* and *Aspergillus niger*. Out of 107 species 05 isolates were selected for further study on the basis of maximum zone of inhibition and broad spectrum activity. The isolated species belong to genera *Strptomyces* according to the Bergeys manual. GA 22 was very active marine actinomycetes which was able to produce variety of enzymes and utilize number of sugars.

Anupama P. Pathak and Sardar Aparna 2012 isolated haloarchea producing **carotnoid** from solar saltern of mulund Mumbai Maharashtra India. Total seven efficient strains of carotenoid producing halophiles were isolated and characterize. Of these a gram negative aerobic rod shaped organism SS-12 was selected for carotenoid production. Later it was identified as *Halorubrum species*, it shows luxuriant growth at 20% Nacl concentration. The isolated pigment analysed spectrometrically and revealed that pigment is **Bacterioruberin** type. Carotenoids used against several diseases.

S.Krishnakumar *et al* 2013 cultivated *Dunaliella salina* at various pH, Temperature and salinity found that can shows maximum biomass of algae at 25^0c, pH-9 and salinity 40%. Algal extract synthesizes bioactive compounds 3, 3, 5-trimethyle heptanes, 2,3,4-trimethyl heptanes, 3,3,4-trimethyl heptanes, 2,4,4-trimethyl heptanes extracted by using solvent like butanol, acetone, isopropanol, methnol, chloroform etc. The maximum antimicrobial activity shown by extraction with chloroform and methanol combine. Extract shows maximum activity against gram negative and gram positive bacteria.

Objectives:

- To collect samples aseptically from multipond marine solar salterns of Mulund, Maharashtra.
- Physicochemical analysis of collected Brine sample by standard method.
- Enrichment and isolation of Halophiles from the collected samples.
- Morphological, biochemical and phylogenetic characterization of Halophiles.
- Screening of isolates for efficient carotenoid producer.
- Production and characterization of carotenoid.
- Molecular analysis and deposition of efficient carotenoid producer in international collection center.

Methodology to be adopted:

1. Collection of Brine Sample :-

   Samples will be collected aseptically from multipond marine solar salterns of Mulund (E) (19^{o} 10'12''N, 72 ^{o} 57'18''E) Mumbai. Samples will be filter and stored in refrigerator. Composite brine samples spreaded on nutrient agar with 20% salt in halophile agar and plates will incubate at 35^{0} C for 14 days. After incubation plates will observe and red pigmented colonies will selected for further analysis.

2. Identification of selected isolates:-

   Selected colonies will be subculture on nutrient agar plate with 20% salt and incubate at 35^{0}C. Microscopic and macroscopic features of grown isolates will record. Amongst many pigmented colonies intense red colored fast growing colonies will be selected for further analysis. Gram's staining will perform by Dussalt's modified methods.

3. Biochemical Characterization:-

   Isolates with bioactive compounds potential will be identified using cultural, morphological, biochemical characters.

4. 16S rRNA gene sequencing analysis.
5. To determine phylogenetic relationship among the related isolates16S rRNA sequencing method will be used.
6. Extraction, characterization and production of carotenoids will be carried out using specific media and method.

Importance of study Application :-

Importance and use of carotenoids in food products Commercial food products using carotenoids are expanding, and the greatest demand is in the Asian continent. In Asia, the production red koji dates of hundreds of years and uses the fermentation of rice by Monascus to produce the typical reddish color. These red pigments are also used as food colorants for wine, red soy cheese, meat, and by-products of meat and fish. The French cheese named vieux-pan contains the carotenoid produced by Brevibacterium linens due to its orange-red-brown color that improves the sensory quality of the product.

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International Status:-

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Arvind Kulkarni *et al* 2012 isolated halophile from marine water of Arabian sea beach of Ratnagiri Maharashtra India. It was they tested for its antibiotic producing capacity. The crude extract from isolate showed inhibition against some gram positive human pathogen like *Bacillus subtilis, gram negative pathogen like Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae*. The extract also showed inhibition against common fungal pathogen like *Candida albicans, Aspergillus niger and Penicillum sp*. This investigation showed new hope of solution to tackle against antibiotic resistance arising in common human pathogen.

Anupama P. Pathak and Sardar Aparna 2012 isolated haloarchea producing **carotnoid** from solar saltern of mulund Mumbai Maharashtra India. Total seven efficient strains of carotenoid producing halophiles were isolated and characterize. Of these a gram negative aerobic rod shaped organism SS-12 was selected for carotenoid production. Later it was identified as *Halorubrum species*; it shows luxuriant growth at 20% Nacl concentration. The isolated pigment analysed spectrometrically and revealed that pigment is Bacterioruberin type. Carotenoids used against several diseases.

Proposed work Plan:

A) First Year

- Literature survey.
- Collection of samples.
- Isolation, identification and characterization of halophiles.

B) Second Year

- Extraction, characterization and production of carotenoids.

C) Third year

- Thesis writing
- Paper publication

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